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Exhaled breath hydrogen cyanide as a marker of early *Pseudomonas aeruginosa* infection in children with cystic fibrosis

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ABSTRACT Hydrogen cyanide is readily detected in the headspace above *Pseudomonas aeruginosa* cultures and in the breath of cystic fibrosis (CF) patients with chronic (*P. aeruginosa*) infection. We investigated if exhaled breath HCN is an early marker of *P. aeruginosa* infection.

233 children with CF who were free from *P. aeruginosa* infection were followed for 2 years. Their median (interquartile range) age was 8.0 (5.0–12.2) years. At each study visit, an exhaled breath sample was collected for hydrogen cyanide analysis. In total, 2055 breath samples were analysed. At the end of the study, the hydrogen cyanide concentrations were compared to the results of routine microbiology surveillance.

P. aeruginosa was isolated from 71 children during the study with an incidence (95% CI) of 0.19 (0.15–0.23) cases per patient-year. Using a random-effects logistic model, the estimated odds ratio (95% CI) was 3.1 (2.6–3.6), which showed that for a 1-ppbv increase in exhaled breath hydrogen cyanide, we expected a 212% increase in the odds of *P. aeruginosa* infection. The sensitivity and specificity were estimated at 33% and 99%, respectively.

Exhaled breath hydrogen cyanide is a specific biomarker of new *P. aeruginosa* infection in children with CF. Its low sensitivity means that at present, hydrogen cyanide cannot be used as a screening test for this infection.



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Exhaled hydrogen cyanide is a specific but insensitive biomarker of new *P. aeruginosa* infection in children with CF <http://ow.ly/TdRaF>

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Introduction

Chronic *Pseudomonas aeruginosa* infection has a detrimental effect on morbidity and mortality in patients with cystic fibrosis (CF) [1, 2]. This can be prevented by early detection and treatment [3, 4]. As a significant proportion of patients with new *P. aeruginosa* infection remain asymptomatic [5], microbiological surveillance is vital to ensure that it is not missed. Unfortunately, this is difficult in children as they are often unable or unwilling to expectorate sputum. In such patients, *P. aeruginosa* detection is dependent on cough swabs, which are unreliable [6, 7]; on sputum induction, which is time consuming and expensive [8, 9]; or on bronchoalveolar lavage (BAL), which is invasive and requires a general anaesthetic [10]. The urgent need for a child-friendly, noninvasive method to accurately detect *P. aeruginosa* infection has led to interest in developing a diagnostic exhaled breath test.

The most extensively investigated biomarker of *P. aeruginosa* that could be used in an exhaled breath test is hydrogen cyanide. It has been known for more than a century that *P. aeruginosa* produces nonvolatile cyanide ions [11]. More recently, selected ion flow tube mass spectroscopy (SIFT-MS) has been used to detect and analyse volatile hydrogen cyanide released into the gas phase by cultures of *P. aeruginosa* [12]. The *in vitro* conditions affecting hydrogen cyanide release have been established [13, 14] and it is known that *P. aeruginosa* is the only organism frequently found in the CF lung that produces hydrogen cyanide [15, 16]. *In vivo* studies have demonstrated elevated exhaled breath hydrogen cyanide in both children and adults with CF and chronic *P. aeruginosa* infection, compared to controls [17, 18]. These studies demonstrated that hydrogen cyanide is a biomarker of chronic *P. aeruginosa* infection. As designed, they could not reveal if hydrogen cyanide was an early marker of *P. aeruginosa* infection, which is necessary if it is to be used as a screening tool. The SPACE (Sensitivity and Specificity of *Pseudomonas aeruginosa* Detection Using the Hydrogen Cyanide Concentration of Exhaled Breath) Study was designed to investigate this. The aims of the SPACE Study were 1) to investigate if breath hydrogen cyanide is a biomarker of early *P. aeruginosa* infection in children with CF and 2) to collect prospective data on the incidence rate of new *P. aeruginosa* infection in children with CF who were previously free from *P. aeruginosa* infection.

Methodology

To achieve the study's aims, exhaled breath hydrogen cyanide concentration had to be measured in a group of children with CF around the time of a new *P. aeruginosa* infection. As the onset of infection cannot be predicted, a large cohort of children who were free from *P. aeruginosa* infection was recruited on the presumption that a proportion would acquire *P. aeruginosa* during the study. At each CF outpatient appointment, in addition to the respiratory microbiology sample taken as part of routine care, an exhaled breath sample was collected for hydrogen cyanide analysis and a clinical details questionnaire completed. At the end of the study, the microbiology results were compared to the breath hydrogen cyanide concentrations and the clinical questionnaire results.

Inclusion criteria

The inclusion criteria were: 1) children 2–16 years old with CF; 2) no *P. aeruginosa* isolated from the child in the preceding 12 months; 3) all routine CF appointments at CF centres included in the SPACE Study; and 4) all children had to be able to provide an exhaled breath sample into a collection bag.

Sample size

The power calculation was based on the expected rise in exhaled breath hydrogen cyanide concentration in those who acquired *P. aeruginosa* [17]. Aiming for a sensitivity and specificity of 95%, it was estimated that exhaled breath hydrogen cyanide concentrations needed to be measured in 46 children at the time of *P. aeruginosa* infection. A retrospective audit at one of the recruiting centres found the rate of new *P. aeruginosa* infections to be 0.1 cases per patient-year. The total patient-years required to originate 46 new *P. aeruginosa* cases was therefore estimated to be 460, which was achieved by following 230 patients for 2 years.

Patient recruitment and follow-up

A total of 233 children were recruited from eight paediatric CF centres across the Midlands and the North West of the UK. Their median (interquartile range (IQR)) age was 8.0 (5.0–12.2) years. At each centre, recruitment was open for 6 months but the study start date varied between centres for administrative reasons. The first child was recruited in June 2011 and the last child in October 2012. The study closed at all centres in December 2013. The median duration of follow-up was 2.0 (1.7–2.3) years. 10 children were withdrawn from the study prior to the close date: six transitioned to adult services and four moved to a different area. The data on these 10 children are included in the analysis up to the point at which they were withdrawn.

Collection and analysis of breath samples

At each study visit, the children were invited to inflate a 1-L gas sampling bag made from 70- μ m-thick Nalophan using one long exhalation. Some younger children needed two or three exhalations. Once the bag was filled, the disposable mouthpiece was removed and the bag sealed. The samples were transported to Keele University (Keele, UK) for analysis using a Profile 3 SIFT-MS instrument (Trans Spectra Limited, Newcastle-under-Lyme, UK) [19, 20]. The bags were punctured with a hypodermic needle directly attached to the heated input arm of the SIFT-MS instrument. This enabled the exhaled breath gas to flow unhindered into the analytical region of the instrument. The mean concentrations of water vapour and hydrogen cyanide were analysed over 100 s while operating the instrument in the multiple ion monitoring mode [21]. Informed by previous studies, the bags/samples were heated to 37°C prior to analysis, which was carried out within 24 h of the sample being collected [22, 23]. In total, 2055 breath samples were analysed. On eight occasions, a child refused to provide a breath sample, and on 23 occasions, the breath sample was not analysed within 24 h due to problems with transport or with the SIFT-MS instrument.

Study visits

There was a total of 2086 study visits; the median (IQR) number of visits per patient-year was 4.8 (3.1–5.6). In addition to the breath sample, a clinical details questionnaire was completed by the child/parent at each study visit. This collected data on the child's general state of health, and any change in cough, sputum production, shortness of breath or exercise tolerance. A respiratory microbiology sample was also taken at each study visit as part of the child's normal CF care. A sputum sample was taken from children who were able to expectorate and a cough swab was taken from those unable to expectorate. Induced sputum and BAL samples were not part of the study protocol, and no microbiology samples were taken at the request of the SPACE Study team. Despite this, if such samples were taken at the request of the clinical team, the results were included in the study analysis. To eliminate any bias by knowing from which children *P. aeruginosa* had been isolated, the SPACE Study team was only informed of any positive culture results at the end of the study.

Statistical analysis

The data are presented as median (IQR) values. The Mann–Whitney U-test was used to assess the difference between two groups and the Kruskal–Wallis test was used when there were more than two groups. The *P. aeruginosa* incidence rates (reported as cases per patient-year) were calculated as numerator/denominator. The numerator was the number of children with a new isolate of *P. aeruginosa* during the study period and the denominator was the “person-time at risk”. The person-time at risk was the sum of the time from recruitment to the new *P. aeruginosa* isolate for those who isolated PA and the total follow-up time for those who remained *P. aeruginosa* infection free. The Poisson 95% confidence interval was calculated for the incidence rates and the Chi-squared test was used to compare the significance of two incidence rates. A p-value of <0.05 was deemed significant.

Given the repeated-measures nature of the data, a random-effects logistic model was used to account for the dependency between multiple observations from the same patient. As a guide to the estimated sensitivity and specificity, we dichotomised the predicted probabilities using a cut-off value of 0.5 and then estimated these quantities for clustered data [24]. The area under the receiver operator characteristics (ROC) curve was estimated using the clustered option in the SomersD option in STATA (StataCorp, College Station, TX, USA). To our knowledge, there is no standard method for calculating the Youden index for correlated data and it was therefore not possible to define the optimal cut-off hydrogen cyanide concentration that predicted *P. aeruginosa* infection.

Ethical approval

Ethical approval for the SPACE Study was granted by the Coventry and Warwickshire Research and Ethics Committee (ref. 10/H1211/48).

Role of funding source

The study sponsor had no role in the collection, analysis or interpretation of data; in the writing of the report; or in the decision to submit the paper for publication.

Results

P. aeruginosa status

None of the children recruited into the study had *P. aeruginosa* isolated from their sample for ≥ 12 months. They were further separated into three groups according to their *P. aeruginosa* status: 113 children had never had *P. aeruginosa* grown from their samples (Never group), 99 had last *P. aeruginosa* isolated from their samples >12 months ago and were not receiving nebulised anti-*P. aeruginosa* therapy at

study entry (Free From (FF) group), and 21 had last had *P. aeruginosa* isolated from their samples >12 months ago but had continued to receive prophylactic nebulised anti-*P. aeruginosa* therapy (Free From But Treated (FFT) group). All those in the FFT group had previously been diagnosed with chronic *P. aeruginosa* infection according to standard criteria [25]. The median age was lowest in the Never group and highest in the FFT group (table 1).

Incidence of new P. aeruginosa isolates

P. aeruginosa was isolated from 71 children during the study (29 in the Never group, 31 in the FF group and 11 in the FFT group). The median (IQR) recruitment ages were similar for those who subsequently grew *P. aeruginosa* and for those who remained *P. aeruginosa* infection free: 7.7 (4.4–12.4) versus 8.2 (5.1–12.1) years, respectively ($p=0.71$). The overall incidence (95% CI) of new *P. aeruginosa* isolates was 0.19 (0.15–0.23) cases per patient-year. The incidence rate (95% CI) was higher in the FFT group than in the Never and FF groups combined: 0.41 (0.20–0.73) versus 0.17 (0.14–0.22) cases per patient-year, respectively (Chi-squared 7.41, $p=0.007$) (table 1). The percentage of patients remaining *P. aeruginosa* infection free at the study end was 74% in the Never group, 69% in the FF group and 48% in the FFT group (figure 1).

Recruiting centres

Table 2 shows the study activity and *P. aeruginosa* incidence rates in the eight centres. As we were comparing the incidence rate of new *P. aeruginosa* infection between centres, we excluded children in the FFT group as they had all previously been diagnosed with chronic *P. aeruginosa* infection and may only have been culture negative due to nebulised anti-*P. aeruginosa* prophylaxis. The proportion of children remaining *P. aeruginosa* free at the study end varied from 56% (centre 3) to 83% (centre 7). Centre 4 had less frequent routine outpatient appointments and, therefore, a lower number of study visits per patient-year.

Pairing of study visits and P. aeruginosa culture results

57 of the 71 children from whom *P. aeruginosa* was isolated had a study visit (with completed clinical details questionnaire and an analysed breath sample) on the day of the positive *P. aeruginosa* culture. In the remaining 14 children, the sample from which *P. aeruginosa* was isolated was not taken at a study visit; eight were postal samples, four were taken at an unscheduled hospital visit and two took place at visits of which the study team were unaware. In these 14 children, the gap between the positive culture and the next study visit was 1–5 weeks, and so the breath analysis and symptom questionnaire data were not used in the subsequent analysis.

Symptoms at the time of P. aeruginosa isolation

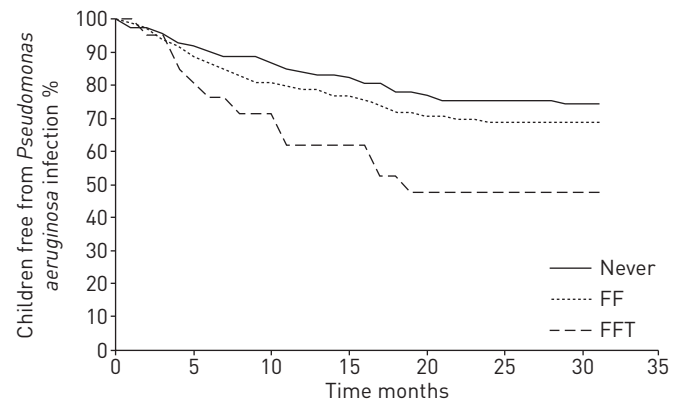
33 (58%) out of 57 children were symptomatic at the time of *P. aeruginosa* isolation. Of these, 30 (91%) had increased cough, 16 (48%) had increased sputum volume, nine (27%) had increased shortness of breath and seven (21%) had reduced exercise tolerance. Only 12 (36%) symptomatic children were described as “unwell” or “less well than normal”.

TABLE 1 *Pseudomonas aeruginosa* incidence rates, study activity and age according to *P. aeruginosa* status

| <i>P. aeruginosa</i> status | Recruits | Age years median (IQR) | Study activity | | <i>P. aeruginosa</i> incidence cases per patient-year (95% CI) |
|-----------------------------|----------|------------------------|----------------|--------------------------------------|--|
| | | | Total visits | Visits per patient-year median (IQR) | |
| Never | 113 | 6.4 (4.0–10.9) | 962 | 4.5 (3.1–5.4) | 0.15 (0.10–0.22) |
| FF | 99 | 8.1 (5.3–12.5) | 910 | 5.0 (3.7–5.6) | 0.19 (0.13–0.27) |
| FFT | 21 | 13.1 (10.6–15.5) | 214 | 5.9 (3.2–6.5) | 0.41 (0.20–0.73) |
| Total | 233 | 8.0 (5.0–12.2) | 2086 | 4.8 (3.1–5.6) | 0.19 (0.15–0.23) |

Data are presented as n unless otherwise stated. IQR: interquartile range; FF: Free From; FFT: Free From But Treated.

FIGURE 1 Kaplan–Meier curve showing the proportion of children remaining free from *Pseudomonas aeruginosa* infection during the study period for each *P. aeruginosa* status group. FF: Free From; FFT: Free From But Treated.



Breath hydrogen cyanide concentrations

At the first study visit, there was no difference in the median (IQR) exhaled breath hydrogen cyanide concentrations in parts per billion by volume (ppbv) between the three *P. aeruginosa* status groups: Never, 2.2 (1.3–3.2); FF, 2.3 (1.5–3.0); and FFT 2.4 (1.2–3.5) ppbv ($p=0.58$). The 162 children who remained *P. aeruginosa* free throughout the study provided 1398 breath samples (figure 2). The median hydrogen cyanide concentration was 2.3 (1.3–3.0) ppbv. The median (IQR) difference between an individual's minimum and maximum hydrogen cyanide concentration (intrasubject variation) was 2.8 (1.9–3.5) ppbv. The hydrogen cyanide concentration on the day of *P. aeruginosa* isolation is shown in figure 3 for the 57 children included in this analysis. The first peak of this bimodal distribution contains breath samples from 29 patients and has a median hydrogen cyanide concentration of 2.2 ppbv. This has a similar distribution to that seen in the *P. aeruginosa* negative patients in figure 2. The second peak contains breath samples from 28 patients and has a median hydrogen cyanide concentration of 6.3 ppbv. All the breath samples in this second peak had a hydrogen cyanide concentration >5.0 ppbv. There was no significant difference between the children in the first and second peaks in terms of age or *P. aeruginosa* status. Five of the 28 children in the second peak had a hydrogen cyanide concentration ≥ 5.0 ppbv for up to two visits prior to the date of the positive *P. aeruginosa* culture and the hydrogen cyanide remained >5.0 ppbv in eight children for up to two visits after the positive *P. aeruginosa* culture date.

Modelling longitudinal hydrogen cyanide concentration

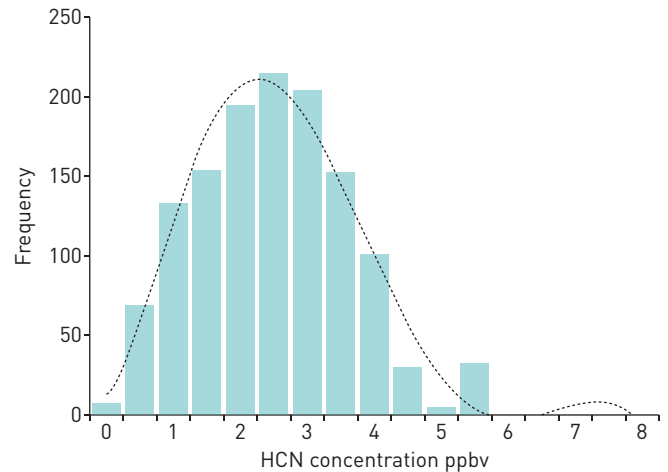
A random-effects logistic model was used to account for the dependency between multiple observations from the same patient. The estimated odds ratio (95% CI) was 3.12 (2.58–3.56), which showed that for a 1-ppbv increase in exhaled breath hydrogen cyanide, we expected a 212% increase in the odds of *P. aeruginosa* infection. The estimated sensitivity (95% CI) was 33% (22–44%) and the estimated

TABLE 2 *Pseudomonas aeruginosa* incidence rates, study activity and age according to recruiting centre

| Centre | Recruits | Age years median (IQR) | Study activity | | <i>P. aeruginosa</i> incidence cases per patient-year (95% CI) |
|--------------|------------|---------------------------|-----------------|---|---|
| | | | Total visits | Visits per patient year median (IQR) | |
| 1 | 24 | 7.6 (4.0–8.0) | 188 | 5.7 (5.3–6.3) | 0.22 [0.10–0.40] |
| 2 | 34 | 8.8 (6.0–12.0) | 348 | 4.9 (4.3–5.2) | 0.18 [0.10–0.29] |
| 3 | 21 | 6.9 (4.9–13.2) | 193 | 4.2 (3.5–4.5) | 0.27 [0.15–0.43] |
| 4 | 53 | 9.1 (5.4–12.5) | 229 | 2.4 (1.9–2.8) | 0.20 [0.12–0.29] |
| 5 | 30 | 9.4 (6.7–12.9) | 306 | 5.2 (4.5–5.5) | 0.13 [0.07–0.25] |
| 6 | 18 | 8.0 (2.4–10.7) | 136 | 4.5 (3.7–5.3) | 0.28 [0.14–0.49] |
| 7 | 36 | 5.2 (3.3–8.5) | 477 | 5.9 (5.2–6.7) | 0.08 [0.04–0.18] |
| 8 | 17 | 8.4 (5.2–12.0) | 209 | 5.5 (4.9–6.2) | 0.22 [0.10–0.40] |
| Total | 233 | 8.0 (5.0–12.2) | 2086 | 4.8 (3.1–5.6) | 0.17 [0.14–0.22] |

Data are presented as n unless otherwise stated. Children in the Free From But Treated group were excluded when the *P. aeruginosa* incidence and antibiotic courses were calculated. IQR: interquartile range.

FIGURE 2 Histogram showing the breath hydrogen cyanide concentrations in samples taken from children who remained free from *Pseudomonas aeruginosa* throughout the study. The dashed line represents the polynomial trend.



specificity (95% CI) was 99% (99.2–99.9%). The area under the ROC curve was estimated as 0.88 (0.83–0.94). This equates to the probability that a child with a new *P. aeruginosa* infection will have higher breath hydrogen cyanide than one who remains free from *P. aeruginosa*.

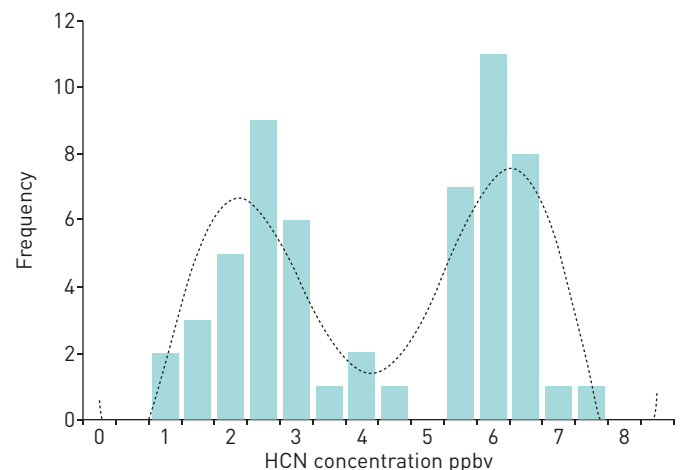
Discussion

The SPACE Study is one of the largest breath analysis studies to be undertaken and is the first to investigate whether exhaled breath hydrogen cyanide can be used as an early biomarker of *P. aeruginosa* infection in children with CF. As well as demonstrating that hydrogen cyanide is a specific biomarker of *P. aeruginosa* infection, it has provided novel prospective data on the incidence of new *P. aeruginosa* infection in children previously free from *P. aeruginosa* infection.

The high specificity with which elevated exhaled breath hydrogen cyanide indicates early *P. aeruginosa* infection is very encouraging and suggests that it may have a role in the identification of this infection. It must also be recognised that this study did not routinely use BAL culture, which is the gold standard method of *P. aeruginosa* detection in nonexpectorating patients. We therefore relied on cough swabs, which only have a sensitivity of 44–82% [6, 7]. It is of particular interest that five children had breath hydrogen cyanide >5.0 ppbv for one or two study visits prior to the date of their positive *P. aeruginosa* culture. Although this is only a small number of children, it shows that in certain situations, exhaled breath hydrogen cyanide may indicate the presence of *P. aeruginosa* infection that has been missed on routine microbiology samples.

The low sensitivity with which elevated exhaled breath hydrogen cyanide predicted *P. aeruginosa* infection was ultimately disappointing. The hydrogen cyanide concentrations at the time of *P. aeruginosa* isolation showed a bimodal distribution (figure 3). The distribution of the first peak was very similar to that seen in the children who remained free from *P. aeruginosa* throughout the study (figure 2). This suggests that in approximately half of the children with a new *P. aeruginosa* isolate, there was little effect on their exhaled

FIGURE 3 Histogram showing the bimodal distribution of breath hydrogen cyanide concentrations taken from children at the time of *Pseudomonas aeruginosa* isolation. The dashed line represents the polynomial trend.



breath hydrogen cyanide. There are a number of possible explanations for this. One factor is the genotype of the infecting *P. aeruginosa*. Our *in vitro* studies have shown that *P. aeruginosa* releases hydrogen cyanide into the gas phase, which is detectable in the culture headspace [12]. When multiple *P. aeruginosa* genotypes were analysed, they all produced hydrogen cyanide but there was a >700-fold difference in the maximum hydrogen cyanide concentration produced by the various strains [13]. Although the exact correlation between the *in vitro* headspace concentrations and the *in vivo* exhaled breath hydrogen cyanide concentrations is contentious, it is reasonable to presume that pulmonary infection with one of the low hydrogen cyanide producing strains may not produce enough hydrogen cyanide to be detected in exhaled breath.

Another factor that may lead to low sensitivity is the methodology of the hydrogen cyanide analysis. The SPACE Study involved the analysis of breath samples taken at multiple CF centres spread over a wide geographic area. Therefore, the only practical solution was to collect the breath samples in bags and transport them to the SIFT-MS instrument at Keele University for analysis. When bag samples are pre-warmed to 37°C and analysis occurs within 24 h of collection, there is good correlation between these “offline” hydrogen cyanide concentrations and the “online” results obtained by the individual breathing directly into the SIFT-MS instrument [22]. Despite the good correlation, offline concentrations are lower, which increases the chance of false-negative measurements. The technology to allow accurate hydrogen cyanide analysis at even lower concentrations, and the use of smaller and more portable devices continues to improve. This raises the possibility of being able to take regular online measurements in the outpatient clinic.

As with all breath trace gas metabolites, there is intrasubject variation in exhaled breath hydrogen cyanide concentrations [17]. Factors that contribute to this include diet and the time of day the sample is taken [26]. Although the children in the SPACE study were requested not to eat or drink for 30 min prior to providing an exhaled breath sample, it was not possible to accurately confirm this and due to the different timings of the CF clinics, it was not possible to standardise the sample time. Other factors that may have affected the sensitivity are the use of mouth-exhaled rather than nose-exhaled breath samples and the effect of younger children using multiple exhalations to fill the bag [18, 27].

Although the UK CF Trust Patient Registry provides data on the prevalence of chronic *P. aeruginosa* infection, it does not provide data on the incidence of new *P. aeruginosa* infection. We believe that the SPACE Study is the first to provide this data. Interesting differences were seen between the *P. aeruginosa* status groups and the different centres. The higher incidence of new *P. aeruginosa* infection in the FFT group compared to the Never and FF groups is not surprising since these children had all been started on long-term nebulised anti-*P. aeruginosa* treatment after being diagnosed with chronic *P. aeruginosa* infection. It is generally assumed that once chronic *P. aeruginosa* infection is confirmed, the organism will continue to be isolated from the patient [28]. The results in the FFT group demonstrate that when such patients are commenced on nebulised anti-*P. aeruginosa* treatment, suppression of the infection may result in *P. aeruginosa* not being isolated for long periods. Therefore, these patients no longer meet the criteria for chronic *P. aeruginosa* infection. How they should be classified on national databases is also open to debate. We were interested in further investigating the variation in *P. aeruginosa* incidence rates between the centres. The exact infection control practice at each centre was clarified but no single practice could be found to explain the difference in *P. aeruginosa* incidence rates. Other factors that may have influenced the incidence rates include the number of patients at each centre, the age of the patients, the model of care at each centre, the severity of the children’s lung disease, and the social interaction and behaviour of the patients and their families.

Summary

This study has demonstrated that exhaled hydrogen cyanide is a specific biomarker of new *P. aeruginosa* infection in children with CF. Its low sensitivity means that at present, hydrogen cyanide cannot be used as a screening test for early *P. aeruginosa* infection. With continued advances in the sensitivity of SIFT-MS technology, and the development of smaller and more portable instruments, this may change in the future. The SPACE Study has also provided novel prospective data on the incidence of new *P. aeruginosa* infection, which may prompt further research on infection control measures and help inform the design of future trials, particularly in relation to *P. aeruginosa* eradication.

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